

Enzyme activity profiles of four different decay-strategy fungi cultivated on birch wood and barley straw

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Tiivistelmä — Referat — Abstract <p>Fungal wood-decayers play an important role in the recycling of biomass and circulation of nutrients in nature. Fungi are capable to convert cellulose, hemicellulose, pectin and lignin, by the action of carbohydrate-acting enzymes (CAZymes) secreted and also by non-enzymatic reactions, depending on the ecology and decay strategy of the fungus.</p> <p>In the present study, four Basidiomycota fungi with different decay strategies were studied to compare their enzyme activity profiles. The white rot fungus <i>Phlebia radiata</i>, brown rot fungus <i>Fomitopsis pinicola</i> and "grey rot" fungus <i>Schizophyllum commune</i> were cultivated on birch (<i>Betula pendula</i>) wood pieces for twelve weeks, whereas the litter-decomposing fungus <i>Coprinopsis cinerea</i> was cultivated on cut barley (<i>Hordeum vulgare</i>) straw for six weeks. All fungi were also cultivated on liquid medium (malt extract 2%) for four weeks. Laccase, manganese peroxidase (MnP), β-glucosidase, xylanase and endoglucanase activities were followed weekly by measuring the absorbances on 96-well plates. The pH and the production of organic acids at each time point were also followed.</p> <p>The results showed that <i>P. radiata</i> produced high laccase and MnP activities. Additionally, high amounts of succinic acid in the aqueous phase of the solid-state cultivations were detected. <i>F. pinicola</i> had a notable production of xylanase activity on birch, in contrast to the moderate β-glucosidase and endoglucanase activities observed on the same substrate. <i>S. commune</i> was a strong producer of β-glucosidase, but especially xylanase activity on solid substrate. Lastly, the litter-decomposer <i>C. cinerea</i> seemed to have a poor performance in enzymatically decomposing the lignin portion from barley straw, whereas a preference on hemicellulose decomposition was observed. Overall, the results indicated the ability of the studied fungi in decomposing the components of the plant cell wall to different extents according to their decay strategy, which is key in the understanding of the ecophysiology of wood-decay and litter-decomposing fungi, and the potential of fungal enzymes for biotechnological applications.</p>			
Avainsanat — Nyckelord — Keywords Lignocellulose, wood-decay fungi, CAZymes, <i>Phlebia radiata</i> , <i>Fomitopsis pinicola</i> , <i>Schizophyllum commune</i> , <i>Coprinopsis cinerea</i>			
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1. Introduction

Lignocellulose is one of the most abundant renewable resources on Earth (1). The recycling of lignocellulosic material in nature is carried out by microorganisms, and saprotrophic fungi play an important role in the cycling of nutrients due to their capacity to convert the complex biopolymeric carbohydrate polysaccharide components of the plant cell wall into sugars that are easier to metabolize (1–4).

Plant cell walls consist of a complex array of polysaccharides and lignin (1–4). The main structural polysaccharides are cellulose, hemicellulose, and pectin. Cellulose consists of long chains of D-glucopyranoses linked together by β -1,4 bonds. These chains form microfibrils that are linked by hydrogen bonds. Hemicellulose consists of a heterogeneous, highly branched polymer with diverse chemical structures depending on the plant species, and this complexity demands an array of specific enzymes to cleave hemicellulosic substrates. Hemicelluloses include, among others, xylan, mannan, β -glucan and xyloglucan (5,6). Pectin has also a variable structure according to the plant species and cross-linking to cellulose and hemicelluloses. Lastly, lignin is a very recalcitrant material consisting of mainly ether-linked aromatic and phenolic subunits, forming a highly variable, plant species and tissue-specific heteropolymer. The phenylpropanoid subunits and partially tightly-joint structure of lignin makes it challenging to depolymerize (1,2,7,8).

Basidiomycota fungi, with emphasis on those from the order Polyporales, possess several enzyme-encoding gene sets in their genomes which make them capable to decompose complex substrates such as lignocellulose (9,10). According to the type of decay, saprotrophic Basidiomycota species can be grouped mainly as white rot fungi, brown rot fungi and soil-inhabiting litter-decomposing fungi (1,7).

White rot fungi are well-known decomposers of lignin, and they can also depolymerize cellulose and hemicellulose (1,2,11). White rot fungi possess an extensive set of carbohydrate-active enzymes (CAZymes) that act on cellulose, hemicellulose and pectin, as well as lignin-modifying enzymes (class-II peroxidases) to oxidize and break down the complex lignin polymers. These lignin-modifying enzymes include laccases, class-II peroxidases from several families such as lignin peroxidases (LiPs), versatile peroxidases (VPs), and manganese peroxidases (MnPs), together with other auxiliary enzymes (1,11,12).

Class-II peroxidases, such as LiPs, MnPs and VPs, catalyze the oxidation of high-redox molecules using H_2O_2 as a co-substrate, and are important enzymes involved in lignin decomposition (7). Among them, MnPs are the most prevalent enzymes in white rot fungi and are common in litter-decomposing fungi. MnP enzymes catalyze the oxidation of Mn^{+2} to Mn^{+3} , and the complex formed by oxalate- Mn^{+3} ions may attack, for instance, phenolic substrates (1).

Laccases, in turn, are included in the AA1 family of auxiliary CAZymes. Laccases are phenol oxidases belonging to the multicopper oxidase superfamily (13), and are involved in different physiological processes such as fruiting body formation, spore formation, oxidation of toxic compounds, but due to their abundance in many white rot fungi, laccases have been also linked to lignin decomposition (1). It has been proposed that laccases act in combination with low-molecular-weight phenolic compounds and nitrogen-substituted compounds to oxidize lignin (1,11).

CAZymes are enzymes divided in several families that can be found in the CAZyme database (www.cazy.org) (14). Those enzymes from the family of glycoside hydrolases (GHs), carbohydrate esterases (CEs) and auxiliary activities (AAs) are the main enzymes involved in the cleavage of polysaccharides (15). It is assumed that fungal secreted CAZymes act synergistically in the depolymerization of cell wall polysaccharides. In the set of enzymes produced by Basidiomycota fungi, the breakdown of β -1,4 glycosidic bonds is mainly performed by GH3 family β -glucosidases, which cleave cellobiose into glucose. In GH5 family, there are enzymes such as endoglucanases cleaving internal-chain β -1,4 glycosidic bonds, and also enzymes acting on hemicellulose, such as xylanases, xyloglucanases in families GH5, GH12 and GH74, and endomannases in family GH5 (1,14).

Brown rot fungi depolymerize the polysaccharides present in the plant cell wall, but lignin is barely modified and it is left behind as a brownish residue (1). In contrast to white rot fungi, brown rot fungi have lost their class-II peroxidase-encoding genes, and the depolymerization of carbohydrates is mainly via non-enzymatic Fenton reactions. Several CAZyme sets are also encoded in the genome of brown-rot fungi, which aid in the breakdown of polysaccharides (1,2,10).

Analysis of the genomes of different Basidiomycota wood-decomposing fungi also suggests more than two types of wood-decay (16,17). One clear example is *Schizophyllum commune*, previously classified as a white rot fungus, even though the species has a

limited capacity of decomposing lignin. It has been proposed that *S. commune* uses a non-enzymatic mechanism to decompose the components of the plant cell wall together with CAZymes, following a similar mechanism observed for brown rot fungi (18,19), but definitely showing its own decay pattern.

Litter-decomposers are filamentous fungi inhabiting soil environments including plant residual litter such as small branches, needles, leaves, and small pieces of wood found, for instance, on the forest soil. Litter-decomposing fungi secrete mainly CAZymes, being able to break down cellulose, hemicellulose and pectin. Some of these fungi, like species of *Agaricus* and *Agrocybe*, also produce class-II peroxidases to attack lignin molecules (4,20). However, the litter-decomposer fungus *Coprinopsis cinerea* secretes another low-redox potential peroxidase that is not efficient against lignin but may oxidize and activate phenolic compounds and humic substances (1). Thus, litter-decomposers play an important role in nature in the recycling of nutrients, together with wood-decayers (4).

In this study, the enzyme profiles of four common, saprotrophic Basidiomycota fungi with different lifestyles and decay strategies were compared to find differences and similarities in the extracellular enzymes secreted when the fungi were growing on the selected lignocellulose substrates. The understanding of the main features of each decay strategy and the enzymes secreted by the fungi is the first approach to identify which fungi or their enzymes could be selected for biotechnological processes, such as biopulping, degradation of pollutants, biofuel generation, lignocellulose pretreatment, etc. (7). Additionally, the study also contributes to a better understanding of the ecophysiology of wood decayers and litter-decomposers on their specific substrates.

2. Materials and methods

2.1.Fungal isolates

The Basidiomycota isolates used in the present study were obtained from the Fungal Biotechnology Culture Collection of the Microbial Domain Biological Resource Centre HAMBI (HAMBI mBRC), which is a part of the Biodiversity Collections Research Infrastructure in the Helsinki Institute of Life Science of the University of Helsinki. The isolates belong to the class Agaricomycetes, species *Phlebia radiata*, *Fomitopsis pinicola*, *Schizophyllum commune* and the *Amut Bmut* homokaryon strain of *Coprinopsis cinerea*, and have their own decay strategy (Table 1). The fungal isolates were kept on 2% (w/v) malt extract (Biokar Diagnostics, France) agar (MEA) plates at 25°C in the dark for seven days. The fungi selected have been genome sequenced with annotated genomes

available at the DOE Joint Genome Institute (JGI) MycoCosm genome repository (<https://genome.jgi.doe.gov/mycocosm/home>) (21).

Table 1. Fungal isolates used in this study.

Fungal species	Isolate code	Decomposition strategy	Reference
<i>Phlebia radiata</i>	FBCC 0043	White rot	(22)
<i>Fomitopsis pinicola</i>	FBCC 1181	Brown rot	(9)
<i>Schizophyllum commune</i>	H4-8 A	“Grey rot”	(18)
<i>Coprinopsis cinerea</i>	<i>Amut Bmut</i> P ⁺ B ⁻	Litter decomposer	(23)

2.2. Culture conditions

The fungal isolates *P. radiata*, *F. pinicola* and *S. commune* were cultivated on small pieces of birch (*Betula pendula*) wood (birch sawdust) originating from a single tree felled in spring 2018 in South of Finland, cut to pieces, dried at room temperature, and sieved with a 5 mm mesh size metal sieve to obtain more homogenous particle size distribution in the solid substrate. For *C. cinerea*, air-dried and cut pieces (length about 2 cm) of barley (*Hordeum vulgare*) straw obtained from the 2017 autumn harvest from the University of Helsinki Viikki Campus research farm fields were used as substrate.

The wood pieces and cut barley straw were oven-dried at 60°C for seven days and at 70°C for three days, respectively. Three grams of either wood or straw were weighed into each 100 mL conical glass flask closed with a cellulose stopper and a metal cap. All the flasks were autoclaved at 121°C for 15 minutes (dry run), and 10 mL of 0.5% (w/v) yeast extract solution in Milli-Q water (autoclaved at 121°C for 15 minutes before use) were added to each flask on top of the solid lignocelluloses.

Both solid-state and liquid cultivations were started for each fungal isolate. The solid-state cultivations were started by inoculating one 0.5 cm² sized mycelial plug from pre-cultured fungal MEA plates into each 100 mL flask. For the liquid cultivations, one plug was inoculated in each 250 mL flask containing 50 mL of previously autoclaved 2% (w/v) malt extract (pH 5.5; Biokar Diagnostics, France) (ME) medium. The solid-state cultivations, three parallel flasks for each fungus, were incubated in the dark at 25°C for twelve weeks, and the liquid cultivations were incubated in the same conditions but for four weeks only.

The samples were aseptically taken (1 mL) from the aqueous phase of the cultivations once a week and kept at -20°C for further analysis. Sterile MilliQ-water, 1 or 2 mL, was added to the flasks to keep the moisture and water content stable in the solid substrate. In the case of the liquid cultivations, no water was added after taking the 1 mL sample. Controls without fungi for both solid-state and liquid cultivations were incubated under similar conditions.

In the end of the cultivations (four weeks liquid cultures, twelve weeks birch wood cultures, six weeks barley straw cultures), the mycelia from the liquid cultivations and the solids from the solid-state cultivations were quickly frozen in liquid nitrogen and stored at -80°C. Incubation of the control flasks was finished earlier (nine weeks for birch wood, and five weeks for barley straw) due to contamination. In total, six control flasks were discarded (three from birch wood and three from barley straw).

All cultivations were performed with three biological replicates (three parallel flasks) and the mean value of three biological replicate flasks was reported in the results.

2.3.Enzyme activity assays

Samples were quickly thawed and mixed before measuring the enzyme activity. For those samples that presented agglomerates of suspended wood dust, a five-minute centrifugation step (13 000 g) was also performed prior to the enzyme activity assays to avoid interference during the reading of the absorbances. The enzyme activities are reported as the mean values of three biological replicate cultivation flask samples. In 96-well plate assays, each sample was repeated in three individual wells as technical replicate reactions, with a final volume of 250 µL in each well. In exceptional cases, values were left out of the calculations according to the accepted range of standard deviation observed between the values of the three replicates.

Laccase, manganese peroxidase (MnP), β -glucosidase, endoglucanase, and xylanase activities were determined using 96-well plate methods previously established (5,24) using Tecan Spark M200 multimode microplate reader (Tecan, Switzerland). Polypropylene NUNC™ F microwell 96-well plates with flat bottom (Thermo Fisher Scientific, USA) were used to measure laccase, β -glucosidase, endoglucanase, and xylanase activities. For MnP activity measurements, Costar UV-Transparent microplates were used (Corning, USA). For the samples of *F. pinicola*, laccase activity was measured for the first and last three time points only, and MnP activity was not measured since no

extracellular peroxidase activity was assumed in the cultures due to the lack of class-II peroxidase genes in the genome of the fungus, as were previously reported (1,25,26).

For laccase activity, the formation of the green product as a result of the oxidation of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Sigma, USA) (31.25 mM) in 50 mM sodium malonate buffer (pH 4.5) was followed at 420 nm (24). For MnP activity, the formation of Mn^{+3} -malonate chelates was followed in 50 mM sodium malonate buffer (pH 4.5) at 270 nm. For β -glucosidase activity, 1 mM of 4-nitrophenyl β -D-glucopyranoside was used as enzyme substrate, and the formation of the product *p*-nitrophenol was measured at 400 nm. Endoglucanase activity was determined with 1% (w/v) hydroxyethyl cellulose (HEC, Sigma, USA) as enzyme substrate, and xylanase activity was determined with xylan from beech wood (1% w/v, Sigma) as substrate.

The reactions of xylanase and endoglucanase activities were stopped by the addition of 100 μ L of DNS (dinitrosalicylic acid), after a five-minute incubation step. The reduced sugars released from the substrate reacted with the DNS, forming a red brown product which was followed at 540 nm after boiling (5,24). Standard curves with known concentrations of 4-nitrophenol 0.01 M, xylose 0.01 M and glucose 0.01 M were plotted to estimate the β -glucosidase, xylanase and endoglucanase activities, respectively. Highly concentrated samples were diluted prior to the enzyme activity assays in order to have a better estimation of the activity.

The enzyme activities were tested for the samples from both liquid and solid-state cultivations, except for endoglucanase and xylanase activities, which were measured from samples of the solid-state cultures only. The liquid culture samples were omitted due to the high interference observed with the DNS caused by the free sugars already present in the ME medium. For endoglucanase and xylanase activities, a net estimated activity was calculated by subtracting the absorbances of the control without fungi from the absorbances of the sample. This aided to have a better estimation of the enzyme activities, and to avoid misinterpretations, especially on those cases where estimated activity of the controls overlapped with the estimated activity of the sample.

2.4.Fungal-produced organic acid quantification

The organic acids produced by the fungi on the solid-state substrate were quantified using an UHPLC method previously optimized (27). The thawed culture fluid samples from weeks one, two, four, eight and twelve were centrifuged as previously described (see

Section 2.2), and filtered prior to the injection (2 μ L) through 0.2 μ m-pore-size Chromacol membrane filters (Thermo Scientific, USA) to remove disturbing agglomerates, directly into 0.2 μ L chromatography sample vials with integrated glass insert (VWR, USA). Samples corresponding to the incubated controls of wood pieces without fungi from week one and nine, and controls of straw cuts without fungi from week one and four were also analysed as references. If necessary, the samples were stored in their vials at -20°C for maximum two weeks prior to HPLC runs. The analysis of the samples was performed by Hans Mattila, by using an Agilent 1290 Infinity binary LC 171 system (Agilent Technologies) coupled with a guard cartridge (Agilent Technologies) and reverse-phase Luna C18 column (150 mm by 4.6 mm, 3- μ m particle size; Phenomenex). The column temperature was 40°C, and separation was performed at a flow rate of 0.950 ml min⁻¹ under isocratic conditions by using an eluent mixture consisting of 95% (v/v) of 0.3% H₃PO₄ and 5% (v/v) acetonitrile. The elution of carboxylic acids was followed as absorbance at 210, 260 and 280 nm. Reference standard samples with variable concentrations of a set of organic acids (27) were used to quantify the acids in the samples. The reference compounds were purchased as the highest purity scale commercial products from Sigma-Aldrich (Merck). The amounts of organic acids quantified were presented as the mean value of the three biological replicates.

2.5.Measurement of pH and other analyses

The acidity of the cultivations was followed by measuring the pH of the culture liquids. Samples corresponding to each time point (twelve weeks for birch wood cultivations, six weeks for barley straw cultivations, four weeks for ME cultivations) were measured with an Orion 920A pH-meter (Thermo Scientific, USA) equipped with a glass pH electrode. To carry out the measurements, the pH electrode was submerged directly into the 1.5 mL centrifuge tubes where the samples were stored. The pH-meter was calibrated with buffers of pH 4 and 7 prior to the measurements. The electrode was rinsed with distilled water between every measurement to avoid crossed-contamination of the samples. The results were presented as the mean value of three biological replicates.

Additionally, isolation of total RNA from the solid and liquid cultivations of *C. cinerea* was performed using a protocol previously established (28). This procedure was performed with Mari Mäkinen as part of collaboration of the supervisor's research team in an international project on fungal transcriptomics. Briefly, mycelia from the solid substrate (barley straw) and ME cultivations of *C. cinerea* were milled to powder using

an IKA[®] A11 basic analytical mill (Sigma, USA). The samples were taken from storage at -80°C and kept in liquid nitrogen prior to grinding, and then milled after the addition of liquid nitrogen to the cup of the mill to keep the samples cold all the time. The grounded samples were mixed with 10-15 mL of guanidinium thiocyanate (GIT) extraction buffer, incubated for ten minutes and then centrifuged for ten minutes at 10 000 rpm at 4°C. The aqueous layers were transferred to RNase free tubes and kept at 4°C for 24 h. After this, the extracted samples were pipetted over a 2 mL CsCl solution in 13.2 ml polyallomer ultracentrifuge tubes (Beckman-Coulter, USA) and centrifuged at 33 000 rpm for 21 h at 4°C in an Optima L-90 K ultracentrifuge, using the SW-41 Ti swinging bucket rotor (Beckman-Coulter, USA) at the Viikki Campus Biomolecular Complex Purification unit. The supernatant of the samples was removed after centrifugation, and the RNA pellets were rinsed with DEPC-treated water, and then dissolved in 50 µl of DEPC-treated water. The extraction was performed from three fungal replicate cultures on solid (barley) and liquid (ME) substrates. The quantity and quality of the RNA extracted were assessed by Mari Mäkinen using Agilent 2100 Bioanalyzer (Agilent Technologies, USA) of the DNA Sequencing and Genomics laboratory of the Institute of Biotechnology, University of Helsinki. The six RNA samples were sent on dry ice to DOE JGI (Walnut Creek, California, USA) for further analysis and RNA sequencing, which were not performed yet until the completion of this study. Therefore, the results are not included in this work.

2.6. Statistical analyses

The significance between the changes in the enzyme activities observed at different time points was estimated using a one-way repeated measures ANOVA with the post hoc Bonferroni correction. To estimate the significance between the highest enzyme activities observed per fungus, Student's t-tests were applied with Levene's test to test the homogeneity of the data set. A significance of $P \leq 0.05$ (confidence level over 95%) is indicated for the results. Both one-way repeated measurements ANOVA and Student's t-test were applied using IBM SPSS Statistics 25 software package.

3. Results

3.1. Laccase activity

Laccase activity was high for *P. radiata* cultivated on solid-state lignocellulose substrate (birch wood) compared to the other fungi. Laccase activity reached its maximum point on week three (13.9 nkat mL⁻¹) and then significantly decreased ($P \leq 0.05$) from week three

until week nine (4.4 nkat mL⁻¹) (Figure 1A). On ME medium, the observed maximal activity of laccase was low, 1.5 nkat mL⁻¹, and observed only on the first week of cultivation of *P. radiata*. The activity then decreased until week four.

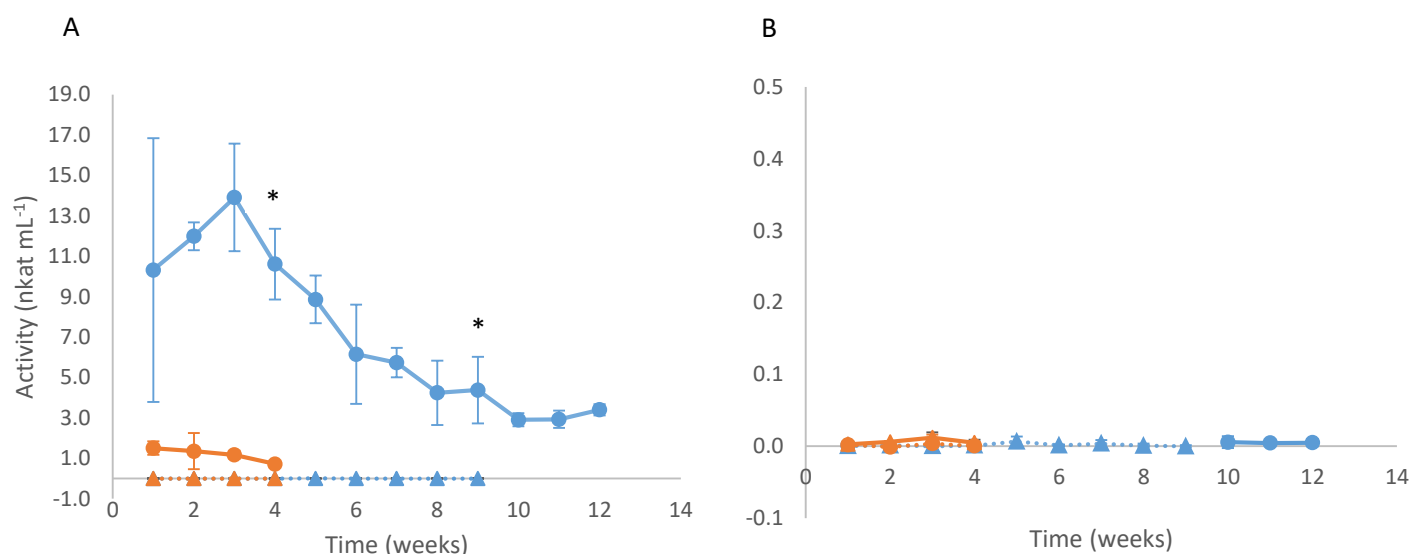


Figure 1. Laccase activities produced by (A) *P. radiata* and (B) *F. pinicola*. Cultivation time on birch: 12 weeks. Cultivation time on ME: four weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3). *: statistically significant changes between time points.

With the other fungi studied, remarkably lower or even zero production of laccase was observed. For the brown rot fungus *F. pinicola*, no laccase activity was detected during the experiment, neither on birch wood nor on liquid ME medium (Figure 1B). For the “grey rot” fungus *S. commune*, very low production of laccase activity was observed during cultivation weeks 4-12 on birch wood whereas on ME medium, no activity was detected in four weeks (Figure 2A). In the litter-decomposing fungus *C. cinerea*, there was a peak of laccase activity on week two (5.5 nkat mL⁻¹) in the solid-state cultures on barley straw. However, no laccase activity was observed on ME medium for the fungus.

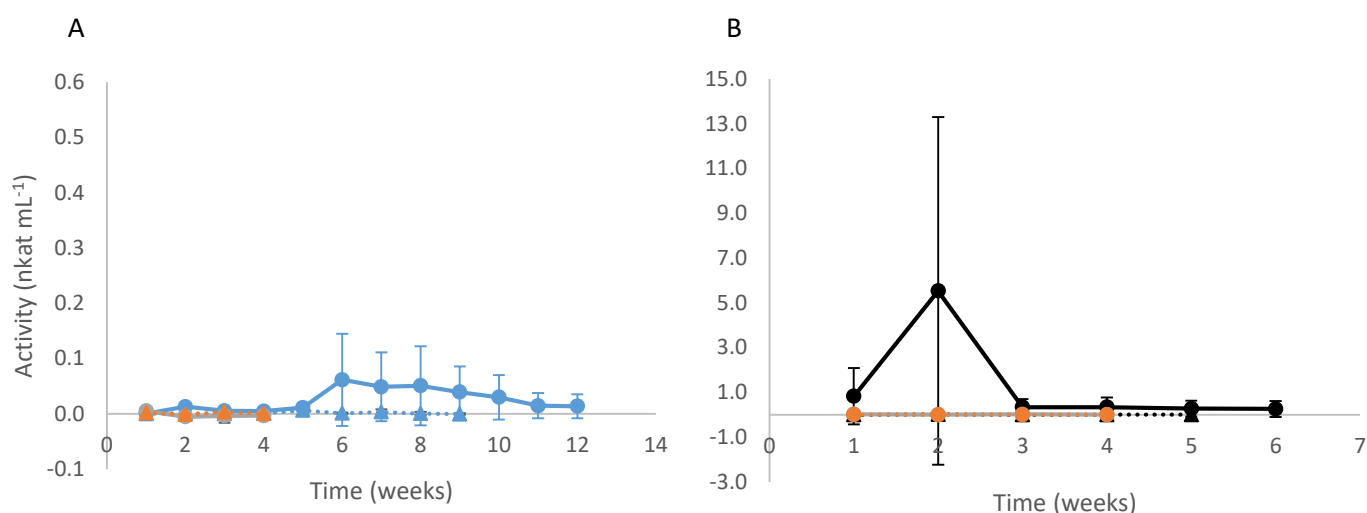


Figure 2. Laccase activities produced by (A) *S. commune* and (B) *C. cinerea*. Cultivation time on birch: 12 weeks. Cultivation time on ME: 4 weeks. Cultivation time on straw: 6 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements from three parallel culture flasks is presented at each time point. Bars indicate the standard error (n=3).

3.2. MnP activity

The white rot fungus *P. radiata* showed a peak of MnP activity (2.4 nkat mL⁻¹) on the second week of cultivation on birch wood (Figure 3A), thereafter the activity significantly decreased ($P \leq 0.05$) until week ten. On ME medium, the highest activity was observed on week three (1.9 nkat mL⁻¹), somewhat later than on birch wood.

In the cultivations of *S. commune* on birch wood, the fluctuating low levels of MnP activity calculated are under the limit of reliable values for the Mn²⁺ oxidation assay reactions (Figure 3B). Regarding *C. cinerea*, the measured MnP activity observed on barley straw was low during the first weeks and increased until the end of the cultivation time on weeks four (1.1 nkat mL⁻¹) and five (2.9 nkat mL⁻¹) (Figure 3C). However, the activity peak on week five is not completely reliable due to the high standard deviation observed between the three parallel cultures. No MnP activity was observed on ME medium neither for *S. commune* nor for *C. cinerea* (Figure 3B and 3C).

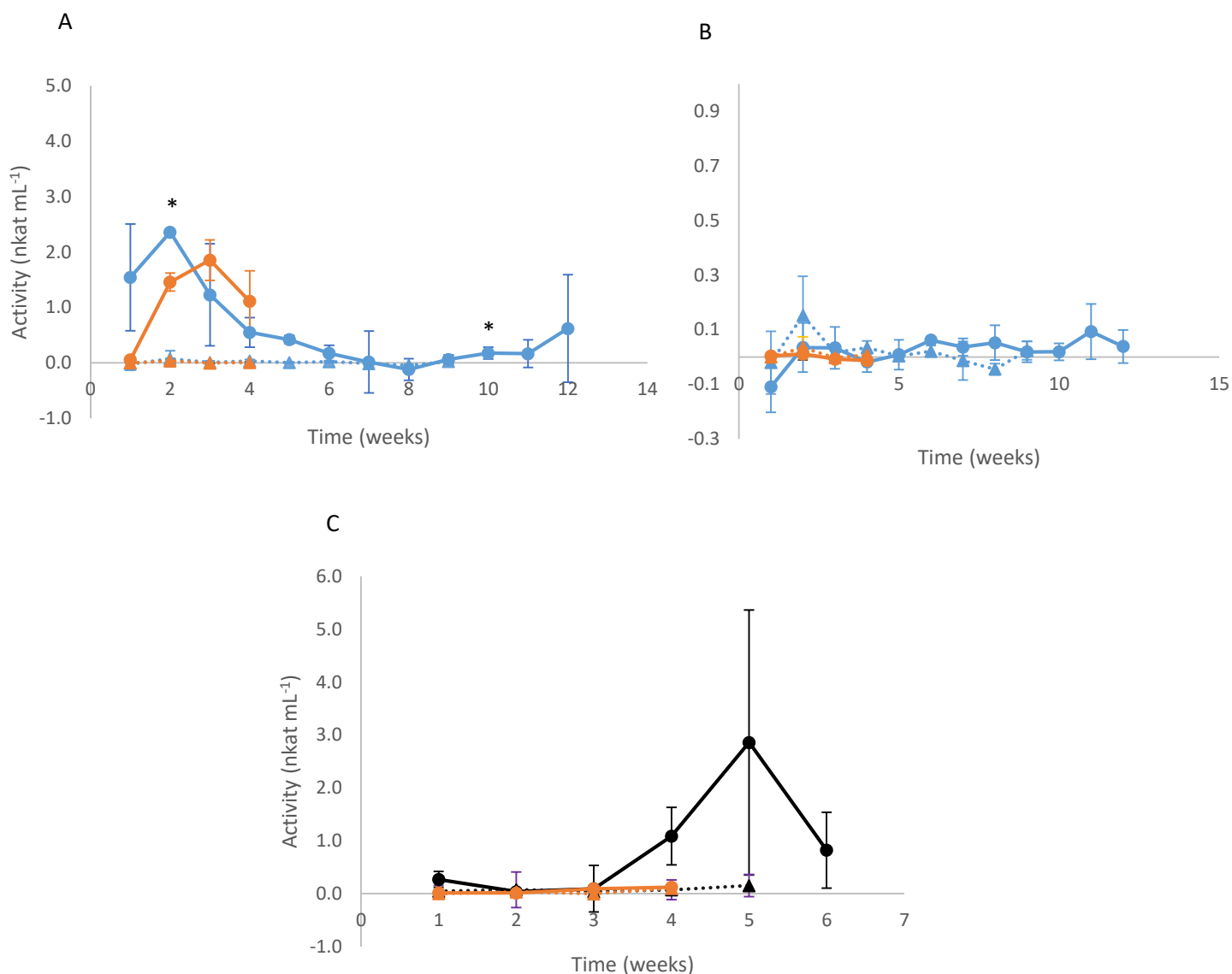


Figure 3. Manganese peroxidase (MnP) activities produced by (A) *P. radiata* (B) *S. commune* and (C) *C. cinerea*. Cultivation time on birch: 12 weeks. Cultivation time on ME: 4 weeks. Cultivation time on straw: 6 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks, if not otherwise stated. Bars indicate the standard error (n=3). *: statistically significant changes between time points.

3.3. β -glucosidase activity

A β -glucosidase activity lower than 1 nkat mL⁻¹ was observed for *P. radiata* during the cultivation time on birch wood, as well as very low levels were observed on ME medium (Figure 4A). For the brown rot fungus *F. pinicola*, on the contrary, β -glucosidase activity significantly increased ($P \leq 0.05$) from the first cultivation week until weeks six and seven (up to 1.5 nkat mL⁻¹), and then decreased to 0.3 nkat mL⁻¹ as it was observed from the tenth cultivation week (Figure 4B). The dashed black line in Figure 4B indicates this drop leaving out the timepoints eight and nine, both of which presented high standard deviations between the three parallel culture flasks and thereby, the mean values are unreliable. Extremely low, near zero mean values for β -glucosidase activity were observed on ME medium for *F. pinicola*.

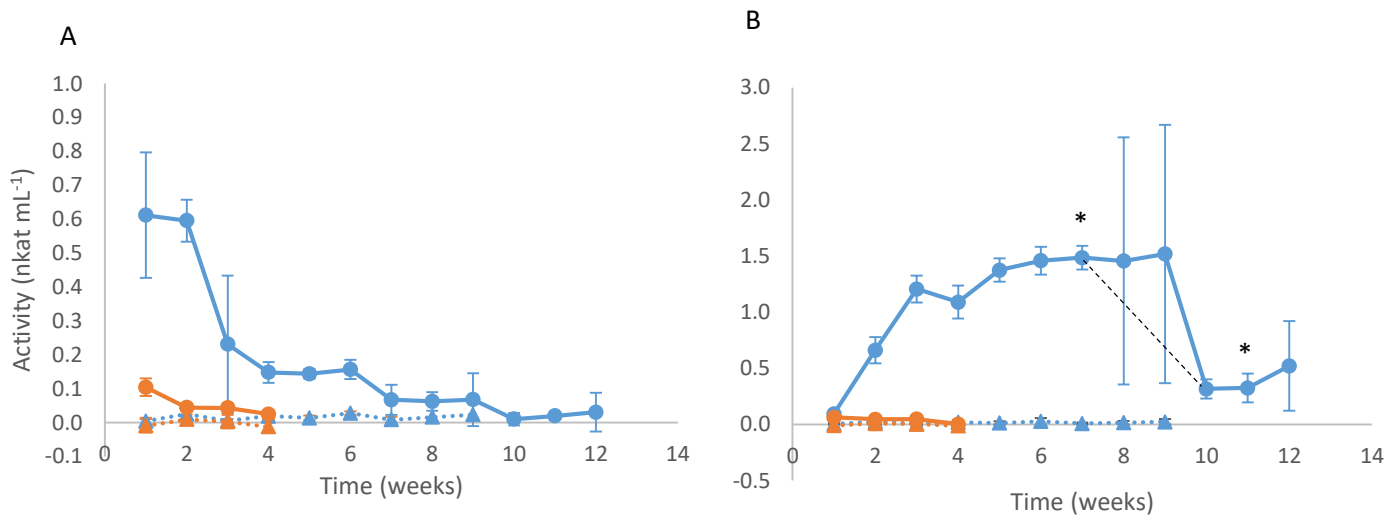


Figure 4. β -glucosidase activities produced by (A) *P. radiata* and (B) *F. pinicola*. Cultivation time on birch: 12 weeks. Cultivation time on ME: 4 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Dashed black line: activity drop. Mean value of the weekly measurements is presented at each time point for three parallel flasks, if not otherwise stated. Bars indicate the standard error (n=3). *: statistically significant changes between time points.

Of all the fungi studied, the highest β -glucosidase activity was observed for *S. commune* in the solid state cultures on birch wood, reaching its maximum on week four (53.6 nkat mL⁻¹) (Figure 5A). Then, the activity significantly decreased ($P \leq 0.05$) until the end of the

experiment to 9.4 nkat mL⁻¹. In contrast, no β -glucosidase activity was observed on ME medium within the four week cultivation for *S. commune*.

For *C. cinerea*, the β -glucosidase activity rapidly increased ($P \leq 0.05$) on barley straw from the value 0.4 nkat mL⁻¹ to 2.0 nkat mL⁻¹ on week three to 1.1 nkat mL⁻¹ in the end of the cultivation on week six (Figure 5B). On ME medium, in contrast, a steadily increasing pattern of β -glucosidase activity was observed for this fungus.

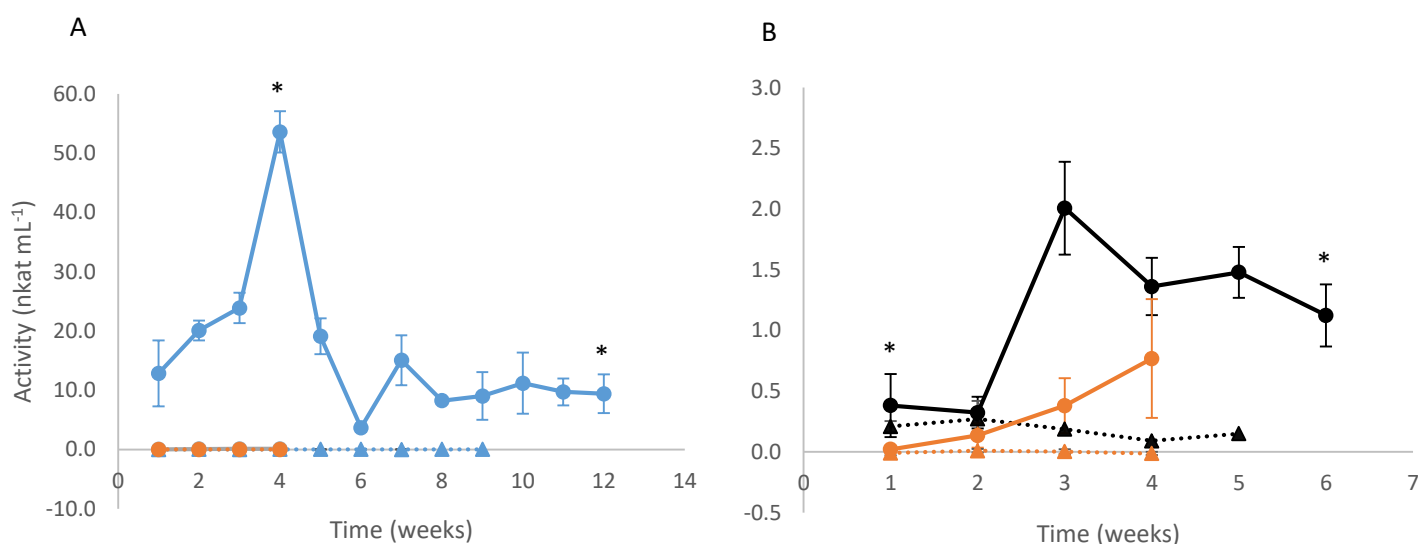


Figure 5. β -glucosidase activities produced by (A) *S. commune* and (B) *C. cinerea*. Cultivation times: birch: 12 weeks, ME: 4 weeks, straw: 6 weeks. Birch (blue circle, solid line). Control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), and control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3). *: statistically significant changes between time points.

3.4. Xylanase activity

The xylanase activity produced by the fungi used in this study was variable, and the most fluctuating activity was observed for *P. radiata* on birch wood (Figure 6A). The net activity estimated (green line) shows peaks on weeks three (4.1 nkat mL⁻¹) and five (4.7 nkat mL⁻¹), but due to the high standard deviations observed between the three parallel culture flasks, no further predictions could be made for production of xylanase activity.

The brown rot fungus *F. pinicola* showed high xylanase activities on birch wood starting from week two and continuing until week nine at very high level (around 40 nkat mL⁻¹) (Figure 6B). From week ten to twelve, the activity decreased close to 12 nkat mL⁻¹. Notable is the high standard deviation of the mean values between cultivation weeks eight to ten, similar to the observations for β -glucosidase activities produced by *F. pinicola* on birch wood (Figures 4B).

Similar to the production of β -glucosidase activities on birch wood, the xylanase activity of *S. commune* was the highest of all the fungi studied (Figure 6C). Xylanase activity occurred in two stages. In the first stage, a significant drop of *S. commune* xylanase activity ($P \leq 0.05$) from week two (447 nkat mL⁻¹) to week six (129 nkat mL⁻¹) was observed. In the second stage, the xylanase activity increased from week six to week seven (575 nkat mL⁻¹) and then significantly decreased ($P \leq 0.05$) to 316 nkat mL⁻¹ on week eleven (Figure 6C).

For the litter decomposing fungus *C. cinerea* on barley straw, xylanase activity was observed from the cultivation week two (4.8 nkat mL⁻¹) until the end of the experiment, with a peak of activity on week five (12.2 nkat mL⁻¹) (Figure 6D).

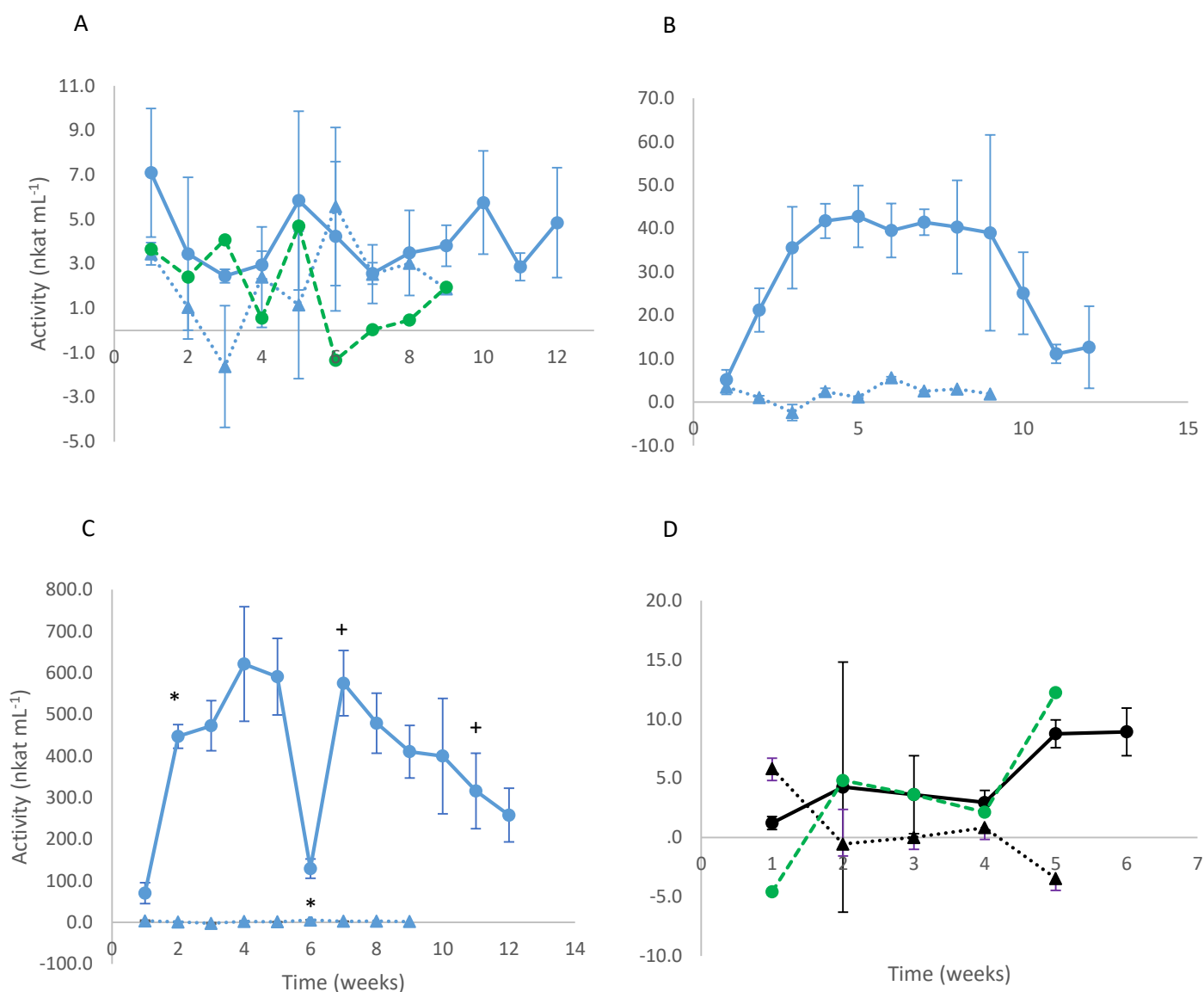


Figure 6. Xylanase activities produced by (A) *P. radiata* (B) *F. pinicola* (C) *S. commune* and (D) *C. cinerea*. Cultivation times: birch: 12 weeks, ME: 4 weeks, straw: 6 weeks. Birch (blue circle, solid line). Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line), net activity estimated (green line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3). * or +: statistically significant changes between time points.

3.5. Endoglucanase activity

For all the fungi studied, an endoglucanase activity level of around 4 nkat mL⁻¹ was observed in their solid-state cultures on birch wood (*P. radiata*, *F. pinicola*, and *S. commune*), and on barley straw (*C. cinerea*) (Figures 7 and 8). Additionally, the activity fluctuated from the beginning until the end of the cultivations. However, for *P. radiata*, the net endoglucanase activity calculated was below the control values (from uninoculated birch wood) almost from the beginning of the cultivations until week five (Figure 7A, green line). Therefore, no endoglucanase enzyme activity was considered to be produced from week one to five. From week seven, the activity values increased to 1.7 nkat mL⁻¹, and a possible increase in the activity was observed until the end of the cultivation.

Like *P. radiata*, the net estimated endoglucanase activity calculated for *F. pinicola* was fluctuating until week five (4.5 nkat mL⁻¹) and thus, is thereafter considered as more or less reliable enzyme activity, which steadily decreased until the last weeks of the cultivations (Figure 7B).

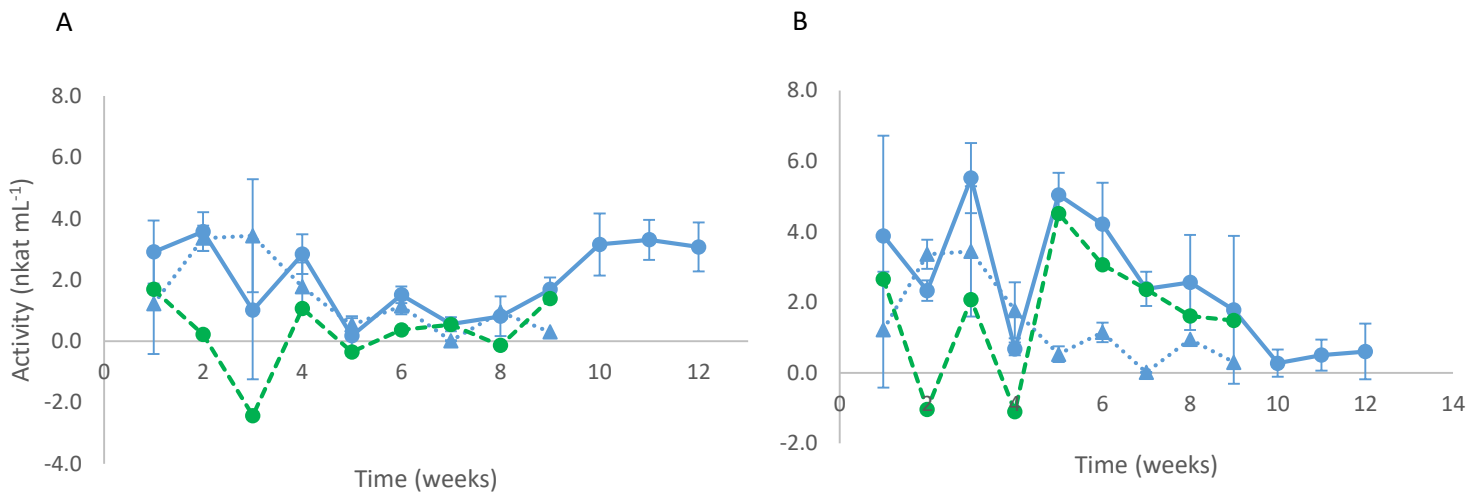


Figure 7. Endoglucanase activities produced by (A) *P. radiata* and (B) *F. pinicola*. Cultivation times: birch: 12 weeks, ME: 4 weeks. The activities of the fungi on ME medium during four weeks of cultivation are also shown. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line), net activity estimated (green line). Mean value of the weekly measurements is presented at each time point for three parallel flasks. Bars indicate the standard error (n=3).

Following a similar pattern as was observed for *P. radiata* and *F. pinicola*, *S. commune* produced endoglucanase activity around week four, and the peak of the activity was reached on week five (3.9 nkat mL⁻¹) (Figure 8A). On the subsequent weeks, the activity dropped to a value around 1.4 nkat mL⁻¹. In the barley straw cultures of *C. cinerea*, endoglucanase activity was produced to a level of 2.9 nkat mL⁻¹ on week five from the first week activities (1.7 nkat mL⁻¹) (Figure 8B).

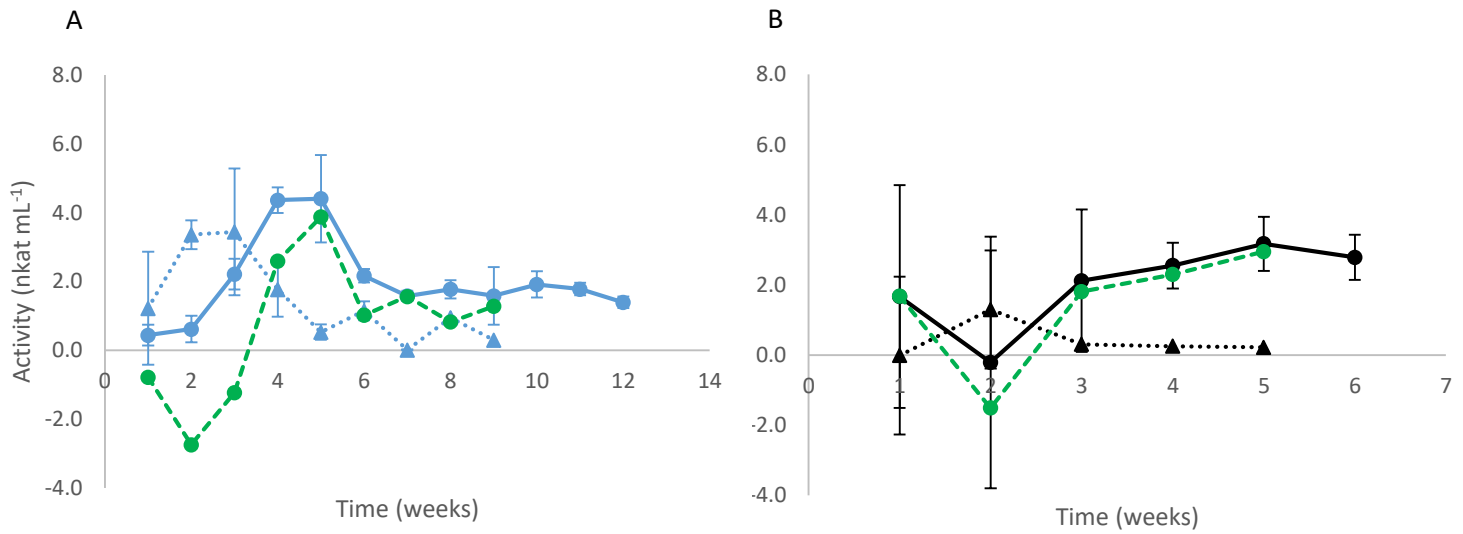


Figure 8. Endoglucanase activities produced by (A) *S. commune* and (B) *C. cinerea*. Cultivation times: birch: 12 weeks, ME: 4 weeks, straw: 6 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line), net activity estimated (green line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3).

Table 2. Highest enzyme activities (nkat mL⁻¹) observed on solid-state lignocellulose cultivations (birch wood and barley straw). The mean of three biological replicates with their standard deviations are presented.

	Laccase	Week	MnP	Week	β -glucosidase	Week	Xylanase	Week	Endoglucanase	Week
<i>P. radiata</i>	13.9±2.7	3	2.4±0.0	2	0.6±0.2	1	7.0±2.9	1	3.6±0.4	2
<i>F. pinicola</i>	0	10	N.A.	N.A.	1.5±1.1	9	42.8±7.1	5	5.5±1.0	3
<i>S. commune</i>	0	6	0	11	53.6±3.5	4	621±138	4	4.4±1.3	5
* <i>C. cinerea</i>	5.5±7.8	2	2.9±2.5	5	2.0±0.4	3	8.9±2.0	6	3.2±0.8	5

N.A.: Non-assayed

*: cultivated on barley straw

A summary of the highest enzyme activities observed is presented (Table 2). The laccase and MnP activities observed for *P. radiata* were significantly higher ($P \leq 0.05$) compared to the same activities observed for *F. pinicola* and *S. commune*, also growing on birch. Regarding β -glucosidase and xylanase activities, *S. commune* produced significantly higher activities ($P \leq 0.05$) compared to *P. radiata* and *F. pinicola*. Endoglucanase activity ranged around 3 to 5 nkat mL⁻¹ in all the fungi studied, and due to the constraints of the method, no further predictions could be made about which fungus produced the highest endoglucanase activity. In the case of the litter-decomposing fungus *C. cinerea*, the fungus produced comparable levels of the enzyme activities measured when cultivated on a different lignocellulosic substrate (barley straw).

3.6. Extracellular pH

A variation in the pH values was observed during the cultivation time of *P. radiata*, *F. pinicola* and *S. commune* on birch wood as well as on ME liquid medium. In the birch wood solid-state cultures of the white rot fungus *P. radiata*, the liquid phase became more acidic with a decrease in the pH from the initial week value of pH 5.1 to the value of pH 3.3 on the third week, and remaining constant until the end of cultivation on week twelve

(Figure 9A). On ME liquid medium, *P. radiata* likewise acidified the medium from pH 4.3 on week one to pH 3.4 on week four (Figure 9A).

In contrast, in the birch wood cultures of the brown rot fungus *F. pinicola*, the initial pH increased to the value of pH 7.6 until the cultivation week ten (Figure 9B). Thereby, the pH of the liquid phase remained neutral, around pH 7, on the last weeks of the experiment. However, *F. pinicola* strongly acidified the ME liquid medium causing a drop of the pH value close to pH 1 already on the fourth week of cultivation (Figure 9B).

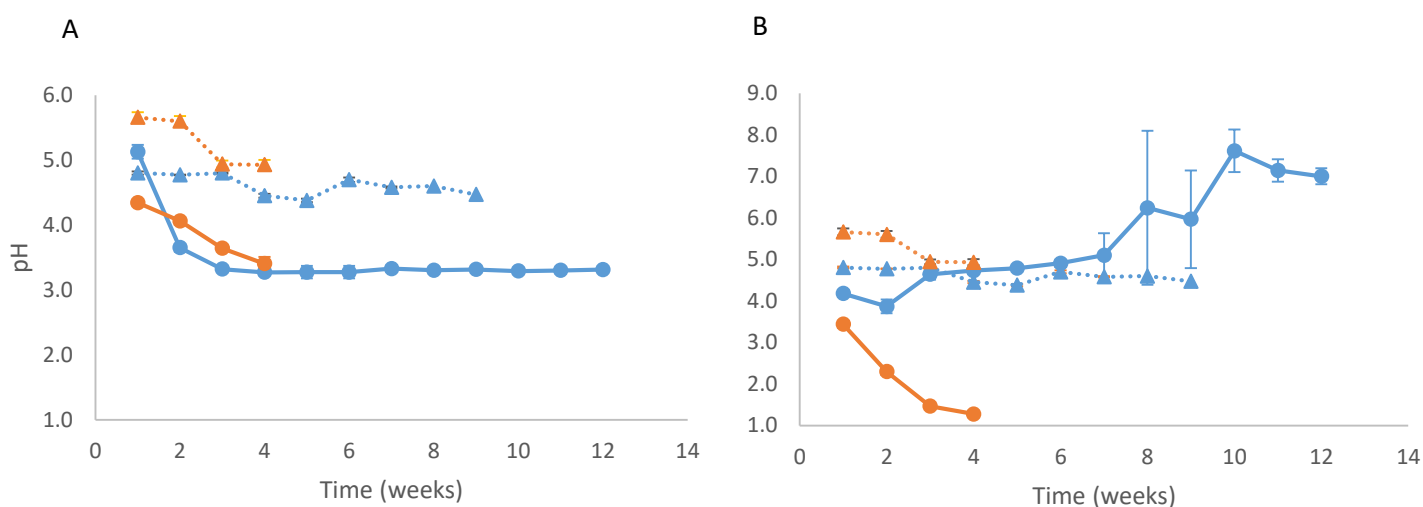


Figure 9. pH values recorded for (A) *P. radiata* and (B) *F. pinicola*. Cultivation times: birch: 12 weeks, ME: 4 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3).

Similar to the observations with *F. pinicola*, an increment of the pH was observed in the liquid phase of the solid birch wood cultivations of *S. commune* (Figure 10A). On the second week, the pH increased from the value pH 6 to neutral, pH 7, and remaining constant until the end of the cultivation time. The pH of the birch control flasks without fungi was ranging around the value pH 4.7 from week one to nine. Likewise on birch wood, *S. commune* increased the pH also on the liquid ME medium, reaching a peak of pH 6.2 on week three and thereby, decreasing to pH 5.6 on week four (Figure 10A). The

ME medium control without fungi ranged from pH 5.7 on the first week to pH 4.9 on the fourth week of incubation.

The pH values in the cultivations of *C. cinerea* on barley straw remained almost constant, at the alkaline pH of the straw substrate (around pH 8) fluctuating between pH 8.8 and 8.5 during the six weeks of the cultivation (Figure 10B). On the liquid ME medium, an increase of the pH to the value 7.2 was observed on cultivation week two, and afterwards somewhat decreasing to pH 5.6 on week four.

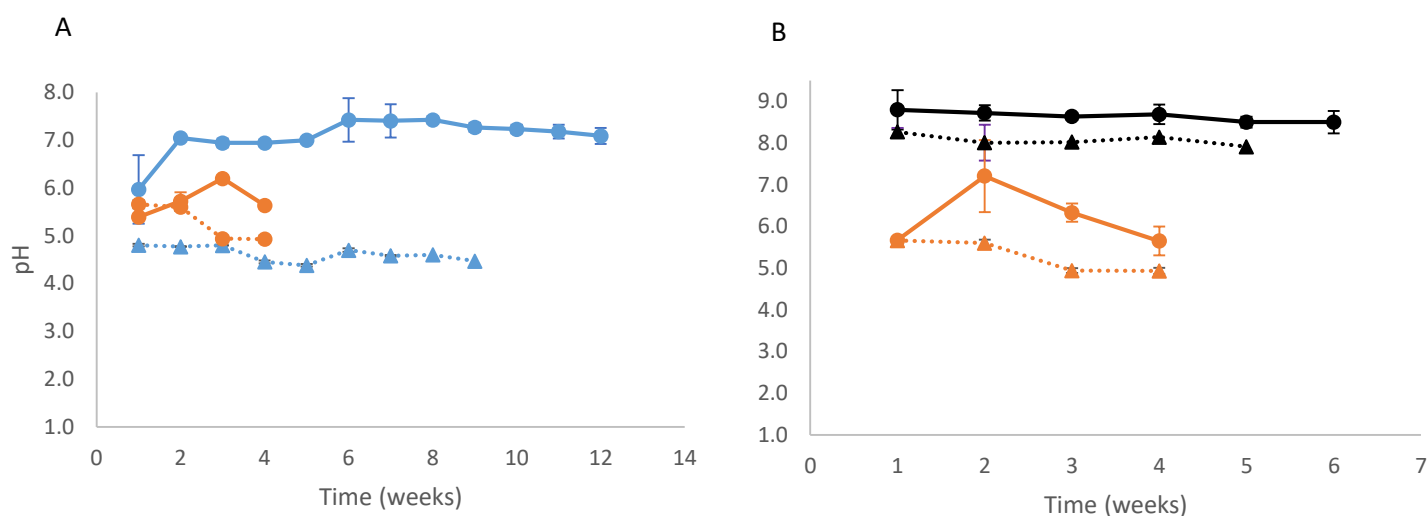


Figure 10. pH values recorded for (A) *P. radiata* (B) *F. pinicola* (C) *S. commune* and (D) *C. cinerea*. Cultivation times: birch: 12 weeks, ME: 4 weeks, straw: 6 weeks. Birch (blue circle, solid line), control birch without fungi (blue triangle, dotted line), barley straw (black circle, solid line), control straw without fungi (black triangle, dotted line), ME (orange circle, solid line) and ME control without fungi (orange triangle, dotted line). Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3).

3.7 Production of organic acids in the solid lignocellulose cultures

The amount of organic acids were quantified from the aqueous phase of the solid-state cultivation samples at chosen timepoints: cultivation weeks one, two, four, eight and

twelve for *P. radiata*, *F. pinicola* and *S. commune*, and weeks one, two, four and six for *C. cinerea* (Figure 11).

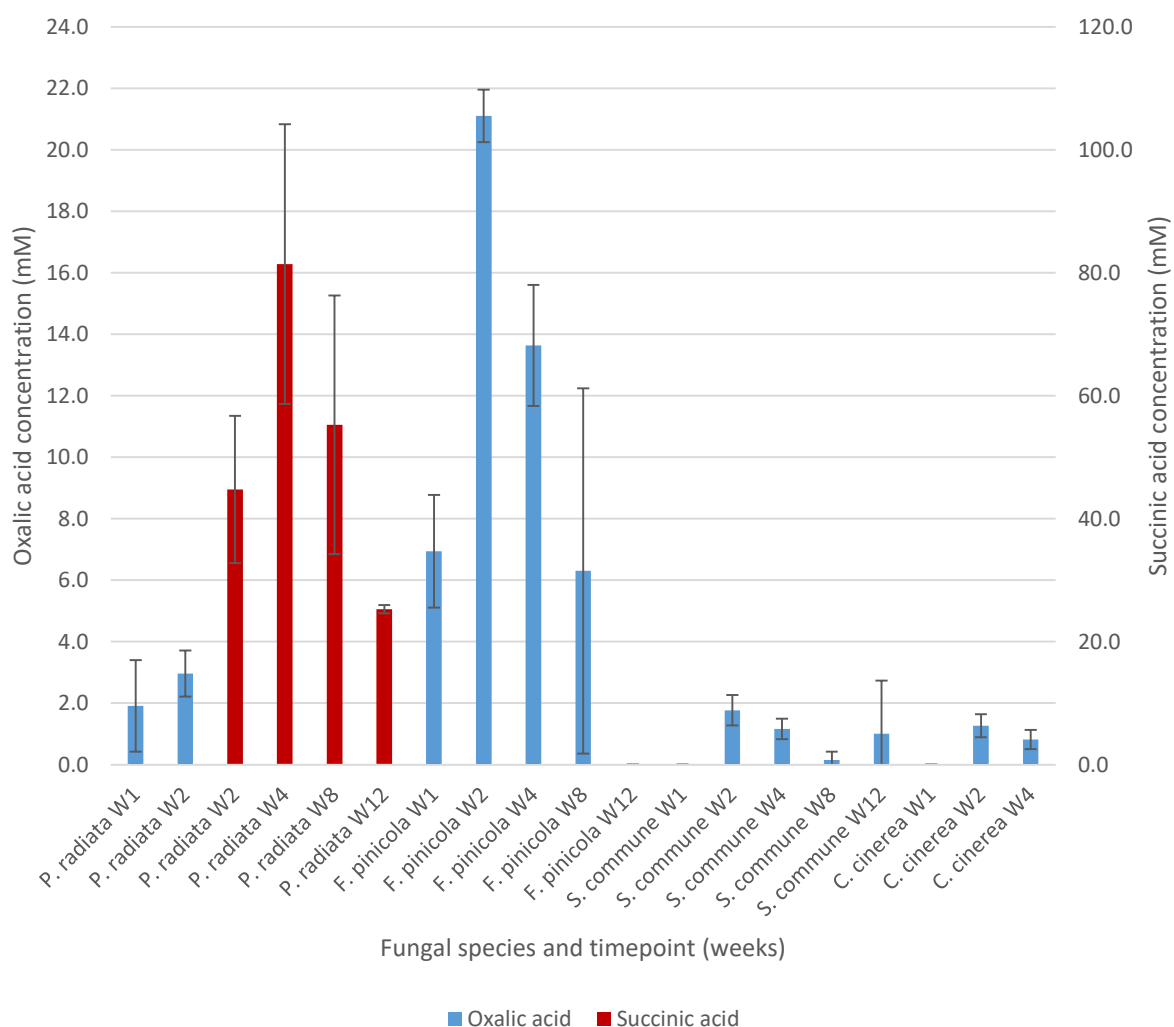


Figure 11. Concentrations of organic acids at different timepoints of the cultivations. W: week. The concentrations of fumaric acid detected in the samples of *C. cinerea* were lower than 0.5 mM and thus, are not displayed in the figure. Mean value of the weekly measurements is presented at each time point for three parallel culture flasks. Bars indicate the standard error (n=3).

P. radiata produced less than 4 mM of oxalic acid during the first two weeks of cultivation on birch wood (Figure 11). Interestingly, high amounts of succinic acid were detected from week two until the end of the cultivation, on week twelve. The concentration of succinic acid increased up to 80 mM on the fourth week and thereby, somewhat decreased on week twelve but still keeping at a high level of 25 mM.

F. pinicola in turn, produced oxalic acid since the beginning of the cultivation on birch wood, up to 21 mM on the second week (Figure 11), and then decreasing in the subsequent timepoints. On cultivation week eight, a high standard deviation between the amounts obtained from the three parallel cultures was observed, indicating that the replicates were not homogeneous. A similar pattern of difference in the pace of fungal growth and metabolism for *F. pinicola* cultivated on birch wood was already observed at the same timepoints in the case of β -glucosidase and xylanase activities (Figure 4B and 6B) as well as in the pH measurement (Figure 9B).

S. commune produced low quantities (less than 2 mM) of oxalic acid during the cultivation on birch wood (Figure 11). Similarly, *C. cinerea* produced less than 2 mM of oxalic acid from week two to four on its selected solid lignocellulose substrate (barley straw). Additionally, less than 1 mM of fumaric acid was detected on week one, two and four in the samples from the cultures of *C. cinerea*. However, a compound eluting in the HPLC analysis similarly as fumaric acid was found in the control samples of barley straw incubated without fungi. In this case, it is not considered that fumaric acid was actively produced by *C. cinerea* in this study.

Consistent with the acidity of the cultivations observed in Figures 9 and 10, a negative correlation (Pearson coefficient, $r = -0.73$) between the total concentration of organic acids present in the samples and the pH detected in the liquid phase of the solid lignocellulose cultivations of the fungi was observed (Figure 12).

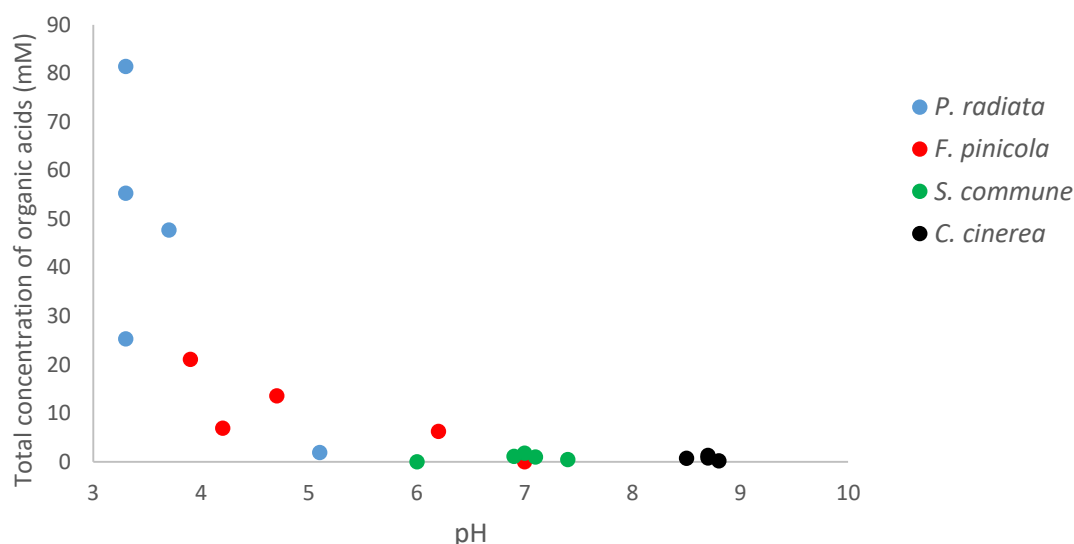


Figure 12. Correlation between the total concentration (mM) of organic acids and the acidity (pH) observed at the selected timepoints of the solid-state cultivations of *P. radiata*, *F. pinicola*, *S. commune* (on birch wood) during 12 weeks (timepoints weeks 1, 2, 4, 8 and 12), and *C. cinerea* (on barley straw) during six weeks (timepoints weeks 1, 2 and 4).

4. Discussion

In this study, the enzyme profiles of four fungi with different decay strategies were analyzed to identify similarities and differences between their enzymatic activities. The white rot fungus *P. radiata*, brown rot fungus *F. pinicola*, and “grey rot” fungus *S. commune* were cultivated for twelve weeks as solid-state cultures using small pieces of birch wood as the substrate for growth, whereas the litter-decomposing fungus *C. cinerea* was cultivated on cut pieces of barley straw for six weeks. All fungi were also cultivated on liquid medium (ME, malt extract medium) for four weeks as stationary cultures at the same incubation temperature (+25 °C) to follow the production of enzymes on a rich carbohydrate sugar containing medium and in the absence of a lignocellulosic substrate.

4.1. Enzyme activity production profiles: *P. radiata*

White rot fungi are well-known producers of oxidoreductases such as laccases and lignin-modifying class-II peroxidases to attack lignin (1,26). Accordingly, the white-rot fungus *P. radiata* showed the highest laccase activity compared to the other fungi cultivated on birch wood in the current study. A similar pattern of laccase activity has been also observed in other studies, when the fungus has been cultivated on wood lignocelluloses a high production of laccase activity has occurred within the first month of cultivation

(24,29). These observations confirm that *P. radiata* is a strong producer of laccase on different substrates. However, the laccase activity obtained on birch was higher compared to the laccase activity on solid spruce wood reported recently by Kuuskeri et al. (2016) (22) and Mali et al. 2019 (29), suggesting a different regulation of the laccase genes when the fungus is growing on birch wood compared to other lignocellulosic substrates previously studied.

The high standard deviation observed on the first week of laccase activity produced by *P. radiata* on birch (Figure 1A) indicated that the production of laccase was not synchronous between the three replicates at the beginning, and that each replicate went at its own pace during the first week. From week two onwards, the amount of laccase produced was more homogeneous between the three biological replicates.

P. radiata produced the highest MnP activity compared to *F. pinicola* and *S. commune*. As observed in previous studies with *P. radiata* on lignocellulose substrates (22,24,26,29), the MnP activity was occurring simultaneously with laccase activity. On ME medium, the pattern of MnP activity was similar to the one observed on solid substrate and also occurred simultaneously with laccase activity, showing the ability of *P. radiata* of producing laccases and MnP in the absence of a lignocellulosic substrate, on a rich carbohydrate medium and in liquid cultures, all features that have been observed previously on the same medium (22,26). These results point to the probability that the *P. radiata* secreted laccase may be involved in other physiological functions of the fungus than participating in decomposition of wood and lignocelluloses, as has been suggested (22).

White rot fungi are able to decompose lignin and polysaccharides present in the plant cell wall, but leaving cellulose as a white residue (3). Therefore, it was not expected to observe a significant production of β -glucosidase activity for *P. radiata* on birch wood. Activities lower than 1 nkat mL⁻¹ were observed in previous studies on spruce wood and in the presence of wood lignocelluloses with the same fungal isolate (22,24,26,29), which is in accordance to the results of this study. Regarding endoglucanase activity observed in *P. radiata*, the enzyme activity profile was difficult to interpret due to the interference caused by the absorbances reported for the birch wood controls without fungi. Apparently, sugars were released from birch wood by the added water during incubation, which interfered with the measurements of endoglucanase activity that is based on the coupled reactions the DNS reagent. However, some endoglucanase activity produced by

P. radiata could be observed after cultivation week three (Figure 7A), just after the action of the enzymes against lignin, indicating that cellulose might be partially utilized after the oxidative attack on lignin units present in the substrate and preventing access to cellulose microfibrils (22).

For xylanase activities produced by *P. radiata*, however, no further predictions could be made due to a high interference of the samples with the birch wood control samples, which was seen in the negative values obtained with the net activity curves. Previously, however, production of xylanase has been observed for the fungus on solid spruce wood cultivations (22,29) as well as in liquid cultures (26).

4.2. Enzyme activity production profiles: *F. pinicola*

Even though *F. pinicola* has three putative genes for laccase production according to genome mining (17), no laccase activity was observed in this study. Similar results were observed in a previous study performed with the same fungus (25,26,29), where no laccase activity was observed on spruce wood or in semisolid liquid cultures. Overall, these observations indicate that the presence of certain genes is not necessarily an indicative of production of enzyme activity, which might be somehow repressed under certain cultivation conditions (25,26,30).

The breakdown of polysaccharides such as cellulose in brown-rot fungi involves mainly non-enzymatic reactions via Fenton chemistry (25). In accordance, moderate endoglucanase and β -glucosidase enzyme activities were observed for *F. pinicola*. In previous studies, *F. pinicola* showed low cellulolytic activity, β -glucosidase and endoglucanase activities, on different lignocellulosic substrates (25,29), which suggests that *F. pinicola* is not a strong producer of cellulolytic enzymes.

Regarding to the depolymerization of hemicellulose, *F. pinicola* produced a high xylanase activity on birch. A high production of xylanase was also observed using the same fungal isolate growing on low-nitrogen asparagine-succinate with spruce wood medium as a substrate (25,26) and a preferred hemicellulose breakdown has been observed in other brown-rot fungi (31). These observations suggest that for *F. pinicola*, the enzymatic attack is an important mechanism to depolymerize hemicellulose together with non-enzymatic reactions.

4.3. Enzyme activity production profiles: *S. commune*

A preference of decomposing polysaccharides has been observed for *S. commune* due to the several CAZy genes encoded in its genome, especially extensive sets of cellulases and hemicellulases (4,19,32). For instance, in cultivations using Jerusalem artichoke as a substrate, *S. commune* produced a high cellulase activity compared to the white rot fungi *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, and the brown rot fungus *Gloeophyllum trabeum* (19). In accordance, *S. commune* was a strong producer of β -glucosidase on birch wood in this study, which is over ten times more compared to the activity observed in the cultures of the white rot fungus *P. radiata* and the brown rot fungus *F. pinicola*. These observations suggest a high expression of β -glucosidase encoding genes in *S. commune* during the cultivation time on birch wood.

Regarding the depolymerization of hemicellulose, *S. commune* showed the highest xylanase activity compared to *P. radiata* and *F. pinicola*. A high xylanase activity produced by *S. commune* has been already observed in a previous study, and the activity was even higher than in a commercial enzyme cocktail produced by the Ascomycota fungus *Trichoderma longibrachiatum* (19). In the current study, extracellular xylanase activity was observed in two cycles, which might indicate that the regulation of xylanase encoding genes varied during the cultivation time. Overall, it was clear that *S. commune* has an efficient machinery for hemicellulose breakdown.

S. commune genome lacks the genes to produce lignin-modifying class-II peroxidases (18). Accordingly, no MnP activity was observed in the cultures of *S. commune* in the present study, neither on birch wood nor on ME medium. Similarly, no laccase activity was detected, even though two laccase genes have been identified in the genome of *S. commune* (18). This may indicate that expression of those genes were somehow repressed under the cultivation conditions used in this study.

4.4. Enzyme activity production profiles: *C. cinerea*

At least eight different laccase genes have been cloned from *C. cinerea* and a few more genes were recognized in a comparative genomic study (9,33). According to this, it was expected to observe laccase production in the solid-state cultures of *C. cinerea* on barley straw. However, no significant laccase activity was observed during the six weeks of cultivation. It has to be noticed that laccases have been linked to different physiological processes besides lignin modification, such as fungal fruiting body formation (33). In this

study, fruiting bodies were observed in *C. cinerea* on the second week of cultivation, and they developed throughout the rest of the cultivation time, so it could be that laccases participated actively in the formation of fruiting bodies and a very low amount of them were secreted to the aqueous phase of the cultivations.

In regard to the peak of laccase activity observed on week two, the activity was not completely reliable due to the high standard deviation and also because of the noise caused by the color of the samples, which affected the accuracy when registering the absorbances from the 96-well plates. However, in other studies performed with *C. cinerea* using rice straw and paragrass as substrates (34,35), moderate laccase activities were observed after two weeks of cultivation, so it might possible that some of the laccase activity observed after two weeks in the present study was reliable.

C. cinerea produces its own type of class-II peroxidases (CiP) that are non-lignin-acting, so called low-redox potential peroxidases instead of MnP or LiP type of peroxidases (1,36). Based on that, it might be that the MnP activity measured was due to moderate production of CiP peroxidase activity during the cultivation time. This was difficult to assure, especially on week five due to high standard deviation between the parallel cultures measured.

Regarding the activity of *C. cinerea* against cellulose, both β -glucosidase and endoglucanase activities were around 4 nkat mL⁻¹, which is lower than the β -glucosidase activity observed in previous studies using rice straw as a substrate (34). However, the increasing β -glucosidase activity on ME medium observed in the current study may indicate that the enzyme activity is involved in other physiological processes and not only in the decomposition of the lignocellulosic material, or that barley straw as substrate is a weaker inducer of β -glucosidase activity.

About the enzyme activity observed against hemicellulose, xylanase was the highest activity observed in *C. cinerea*, especially in the last two weeks of cultivation on barley straw. In a previous study performed with *C. cinerea* on paragrass, the xylanase activity followed a similar pattern as the observed in the present study (35). Taking together, both observations from cellulose and hemicellulose decomposition, it seems that *C. cinerea* had more preference on degrading hemicellulose than cellulose from barley straw.

4.5. Extracellular pH and production of organic acids

It has been proposed that filamentous fungi secrete organic acids to keep a favorable environment for lignin decomposition by chelating metals such as Mn^{+3} ions, keeping acidic conditions on the medium, and producing H_2O_2 to assist in the oxidative decomposition of polysaccharides (1,37–39). In a previous study using the same fungal isolate of *P. radiata* (29), a very low production of oxalic acid was observed on spruce wood, which is in accordance with the current study. However, on birch wood in the current study, an acidification of the liquid phase was observed as apparently caused by the production of high amounts of succinic acid by *P. radiata*. It is possible that on birch wood, succinic acid helped *P. radiata* keeping suitable acidic conditions for the growth of the fungus and enzyme production, and aided in decomposition of wood.

F. pinicola produced oxalic acid during the first four weeks of cultivation on birch wood. The observation was in accordance to pH value around 4.5 observed those weeks. Later, less oxalic acid was detected, and the pH value of the liquid phase accordingly increased. There are previous reports of high production of oxalic acid by *F. pinicola* (25,26,29), which is thought to be involved in the maintenance of a proper environment for the Fenton reactions (25,40). A possible explanation of the increasing pH observed is that the secreted oxalic acid may be attached to the solid substrate as oxalate anions, leaving lower amounts of free oxalic acid on the medium, as was observed recently on spruce wood (29). Also, it is possible that the fungus was growing somewhat less on birch wood, which may have affected the accumulation of oxalate.

During the cultivation time of *F. pinicola*, 1 mL of water was added to keep the moisture of the solid lignocellulose substrate and culture flask. Due to a high uptake of water by the fungi growing into the solid substrates on week nine to twelve, 2 mL were added. It could be that this addition of 2 mL of water to the cultivations may have affected as diluting the acidity of the cultures towards neutrality around pH 7 from week nine to twelve, as was observed. Overall, the performance of *F. pinicola* on ME liquid medium observed as turning to high level of acidity and very low pH values was in accordance with previous observations, where high amounts of oxalic acid were produced by the fungus on liquid medium (25,26).

A low amount of free organic acids was detected in the aqueous phase of the solid cultivations of *S. commune*, and the pH remained around 7. Regarding the growth of *S. commune* on liquid medium (ME), the pH ranged between 5 and 6, which according to

previous observations, is the most suitable pH for mycelial growth of *S. commune* (41). On birch wood, it might be that the lignocellulolytic enzymes of this fungus had a better performance at neutral pH than under more acidic conditions.

C. cinerea was a weak producer of organic acids on solid lignocellulosic substrate and in liquid media. Instead, it may be that the fungus produced metabolites that contributed to alkalizing the media. Additionally, the high pH observed on the solid-state cultivations may be due to the substrate (barley straw) itself displaying pH value around 8.

5. Conclusions

Overall, the results of this study showed that the enzyme activities observed especially in the cultures on birch wood of the white rot fungus *P. radiata* followed the expected pattern with high laccase and MnP activities, and were similar to the observations in previous studies performed with the same fungal isolate. Furthermore, the high amounts of succinic acid present in the aqueous phase of the solid-state cultivations may indicate an influence of the substrate (birch pieces) in the metabolism of *P. radiata*, even possibly induction of metabolic routes not observed on other lignocellulosic substrates. Talking about the brown rot fungus *F. pinicola*, notable production of xylanase activity on birch wood was detected, in contrast to the moderate cellulolytic (β -glucosidase and endoglucanase) activities observed on the same substrate. This suggests that, on birch wood, oxidative Fenton reactions might be the main mechanism of *F. pinicola* in the decomposition of cellulose. The third fungus, *S. commune* was a strong producer of β -glucosidase, but especially xylanase activity was significantly produced on birch wood. It showed the great potential of *S. commune* in breaking down hemicelluloses, which could have a potential in biotechnological applications in the future. Lastly, the litter-decomposer *C. cinerea* apparently had a poor performance in enzymatically decomposing the lignin portion in barley straw, whereas a preference on hemicellulose decomposition was observed. Taken together, the four selected Basidiomycota fungal species studied demonstrated individual enzyme production patterns on birch wood or barley straw, which reflects their differences in decay strategies and saprobic lifestyles on their natural lignocellulosic substrates.

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